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P-53 BINDING PROTEIN CALLED MDMX AND ENCODING SEQUENCES FOR SAID PROTEIN

The present invention relates to molecular biology techniques and especially to their application in cancer research. In particular a method to identify new proteins associating to the p53 tumor suppressor protein, with some examples of cDNAs isolated this way is disclosed. It also relates to the results of said method, which are novel proteins having affinity for p53, as well as genes and other nucleic acid molecules encoding these novel proteins.

In particular the invention also relates to one such a novel protein and its encoding sequences, which protein has homology with the Mdm2 protein and which therefor has been designated as Mdmx.

The process by which a normal cell is transformed into a tumorigenic cell that can grow out to form a tumor is a very complex process in which several independent genetic changes have to take place. These genetic alterations can be roughly divided into two classes. The first comprises the genetic changes that alter the abundancy or properties of a gene product in such a way that it constitutively stimulates cell growth. The second class comprises genetic alterations that inhibit the normal cell growth controlling function of a gene product. An important role in the switch from normal cell to tumor cell is assigned to a protein called p53.

The protein called p53 has properties of both classes. Mutations in the p53 gene as found in human tumors abolish the tumor suppressor activity of the protein. In addition, several mutated forms of the p53 protein can have a dominant stimulating effect on cell growth.

Alterations in the p53 coding sequence are observed in over 50% of all human tumors. This makes p53 mutations the most frequent observed alteration in human cancer.

Many functional studies on p53 indicate that alterations in the p53-controlled functions can be very important for the

genesis and progression of a tumor. Mouse model studies show that either overexpression of a mutant p53 gene or inactivation of the normal wild-type p53 gene results in the development of tumors somewhere between the age of 3 and 6 months.

In humans it has been found that an hereditary form of cancer, the Li-Fraumeni Syndrome, is associated with a germ-line mutation in one allele of the p53 gene. In the tumors the remaining wild-type allele has been lost, indicating the importance of loss of wild-type p53 function for carcinogenesis.

One of the ways in which P53 probably functions is by guarding the integrity of the genome of cells. It can do so in two ways, namely through influencing the cell cycle and/or the process of repairing DNA damage and secondly by influencing the apoptosis pathway.

In the event that cells have received a dose of a DNA damaging agent, like ionizing radiation or some chemicals like cisplatin, p53 can arrest the cell in the cell cycle. The p53 protein may also be involved in the repair of the DNA. The presence of DNA damage results in an increase of the p53 protein levels through protein stabilization. Subsequently, the increased levels of p53 result in the activation of transcription of a set of p53-responsive genes. Two of the most important p53-responsive genes identified sofar are the gene coding for an inhibitor of the cyclin-dependent kinases cdc2, cdk2, cdk4 and cdk6, and a gene called gadd45. The cdk-inhibitor is called $p21^{cip1/waf1}$, and is a member of an expanding family of cdk-inhibitors. These inhibitors inactivate the cdk's by direct binding. The activity of the cdk's, in conjunction with their appropriate cyclin partner, is essential for the progression through the cell cycle. Thus, by increasing the $p21^{cip1/waf1}$ expression, p53 blocks the cell cycle progression and arrests the cells in the G1 phase mainly. The current hypothesis is that this cell cycle delay allows the cell to repair its DNA before the DNA synthesis starts up and introduces mutations at the sites of the DNA

damage. The second p53-responsive gene important in this respect is the gadd45 gene. It has been shown that (over)expression of gadd45 also blocks cell cycle progression and it has been suggested that gadd45 might have a direct role in stimulating the process of DNA repair.

The second process p53 is involved in that is important for maintaining genomic integrity is apoptosis or programmed cell death. Apoptosis is a very ordered, active process that eventually results in the death of a cell. The process is very important in embryogenesis to get rid of superfluous cells. Recently more and more evidence has accumulated showing that interference with the apoptotic pathways can be very important for a cell to become a tumor cell. In addition, a succesfull treatment of tumors might depend on the possibility to induce an apoptotic pathway in the tumor cells. Two *in vivo* studies underscoring the notion that p53 is involved in apoptosis have been most informative. First, it was found that transgenic mice expressing an SV40 largeT mutant which can only bind and inactivate members of the retinoblastoma family($p105^{RB}$, p107, p130) but which has no effect on p53 function, develop tumors of the choroid plexus epithelium, but only very slowly compared to mice expressing the wild-type T-antigen. However, the same transgene can induce the development of rapidly growing tumors in p53-deficient mice. Comparison of the slowly growing and rapidly growing tumors revealed that the slowly growing tumors contained a significant percentage of apoptotic cells, which could hardly be found in the rapidly growing tumors. In another mouse model it was found that treatment of tumors lacking any functional p53 with X-rays or adriamycin hardly affected the tumor growth, while tumors containing a wild-type p53 but otherwise comparable could be eradicated. Analysis of both types of tumors after treatment showed the induction of apoptosis in the wild-type p53 containing tumor cells but not in the cells lacking p53. The mechanism by which p53 induces apoptosis has not been completely solved yet. On the one hand, high levels of p53 can lead to induction of expression of a gene called Bax-1 that has been shown to

stimulate apoptosis. On the other hand, a correlation between the induction of p53-dependent apoptosis and transcription repression by p53 has been found. It could be that both activation and repression of transcription by p53 is necessary to get efficient induction of apoptosis. In addition, cell type specificity in the mechanism by which p53 activates apoptosis is well possible.

The above thus gives an indication on the importance of p53 in suppressing tumor activity and possible mechanisms for said suppression. It also gives an indication of the effects of the absence of functional p53 for instance through mutations in the coding region of p53.

Apart from mutations in the coding region of p53, inhibition of the tumor suppressor function of the wild-type p53 can also occur through an altered-cytoplasmic- subcellular localization. The underlying mechanism for this retainment of the protein in the cytoplasm is unknown.

A third way to abolish the p53 tumor suppressor function is through complexation with another cellular protein called Mdm2. The gene encoding this protein was originally isolated from a mouse double-minute present in a spontaneously transformed Balb 3T3 cell line and shown to be responsible for the transformation. Later, it was found that a protein binding to p53 was exactly the same Mdm2 protein. In the transformed cells Mdm2 probably acts by binding to the p53 protein and inactivating its tumor suppressor function. In addition, it has been shown that binding of Mdm2 to p53 completely inhibits the capacity of p53 to activate transcription of a reported gene containing a p53-consensus DNA binding site in its promoter region. Interestingly, the transcription of the mdm2 gene is also stimulated by increased p53 levels. In that way a kind of feedback loop is established between Mdm2 and p53. Functions for Mdm2 apart from the inhibition of p53 activity are not clear. On the one hand an activity as transcription factor has been suggested, both because of its primary protein sequence that shows some Zinc-fingers possibly involved in DNA binding and because the Mdm2 protein has been found to

associate to the TATA-Binding Protein (TBP). This is the first protein to bind to TATA-containing promoters in the assembly of the basic transcription machinery complex. In addition, it has been reported that Mdm2 can effect the function of another transcription factor important in processes related to cancer, E2F-1. The activity of E2F-1 as a transcription factor is normally controlled by another tumor suppressor protein, the product encoded by the retinoblastoma predisposing gene. Active E2F-1 can activate the transcription of genes encoding proteins that stimulate cell growth, like the myc oncogene. Mdm2-overexpression, mainly through amplification of the gene, has been observed in a significant percentage of certain tumor types, establishing a role for this gene in carcinogenesis.

In conclusion, it is clear that a complete elucidation of the p53-controlled pathways and the identification of genes/proteins involved in these pathways can be very beneficial for understanding the onset and/or development of cancer. One way to understand more of p53 functions is to find out whether p53 has other cellular partners apart from Mdm2 that affect its tumor suppressor activity. An at this moment very widely used method to identify new proteins associating to a protein of interest is the yeast two-hybrid method. With the use of this method in literature two genes have been described coding for p53-associating proteins. However, the molecular functions of these proteins and their putative involvement in carcinogenesis are unknown as yet. Other methods to identify systematically new p53-associating proteins have not been described in literature.

Thus, it it is clear that the development of another way to provide us with genes coding for proteins binding to p53 can be very important for understanding the p53 functions and the process of carcinogenesis. The present invention provides a method to arrive at proteins binding to p53 and the elucidation of their encoding genes.

One aspect of the present invention is the application of a method partly known in itself for the isolation of

associating proteins for the identification of (new) p53-associating gene products.

A first step in the methods according to the invention is the high level expression of the p53 protein in bacteria, with subsequent purification and radioactive labelling of the p53 protein.

A second step is the use of the labeled p53 protein as a probe to screen cDNA expression libraries to pick up associating proteins and subsequently the isolation of the encoding cDNAs.

In a further aspect of the invention the resulting nucleic acids (such as cDNA's) encoding the novel associating proteins and the proteins themselves are provided.
In figures 2a and 2b partial sequences of novel nucleic acids encoding such novel proteins are given. Figure 3 gives a novel cDNA sequence encoding another such novel protein which we have called Mdmx.

This last one is called Mdmx because of its homology to Mdm2.

The longest cDNA isolated sofar is 1701 nucleotides, and contains an open reading frame of 489 amino acids, exactly the same as the mouse mdm2 open reading frame.

The cDNA given in figure 3 is of mouse origin. It will be clear that using the present invention other mammalian species will be shown to have a counterpart of the Mdmx gene. These counterparts are of course included in the present invention. Especially because of the recently developed techniques such as PCR, it will be within the skilled man's ability to identify these counterparts and to design functional equivalents and/or fragments of the novel gene and its products.

It can be seen in fig. 4 that especially the N-terminal part of the Mdmx protein is very similar with the Mdm2 protein. It has been shown that the N-terminal 100 amino acids in the Mdm/*2 protein are essential for the binding to the p53 protein. This result indicates a certain conservation in p53 binding domains in different proteins, suggesting that other

proteins might contain such a domain as well. Furthermore, a strong conservation of two metal-binding domains in the C-terminal halve of the protein is found. This conservation suggests an important function for this part of the protein, possibly in binding to DNA/RNA or to other proteins.

The third conserved motif is a possible nucleotide binding site (GKT) at amino acid positions 451-453. -- (Fig 4; amino acid sequence of the putative Mmdx protein, and a comparison with the mouse and the human Mdm2 protein)

All the applications which have and can be envisaged for Mdm2 proteins or their encoding nucleic acids or parts thereof can of course be applied for Mdmx as well.

For instance, it can be envisioned that the Mdmx gene product(s), like the Mdm2 potein, has a role in certain types of cancer. Therefore, the status of the mdmx gene and the expression level can be monitored. This can be performed in several ways. First, the putative overexpression can be a result of gene amplification. This can be investigated by Southern blotting. We have already performed a pilot study to examine possible amplification of the mdmx gene in primary neuroblastomas and in neuroblastoma cell lines. Equal amounts of paired DNAs of normal and tumor tissue from a patient were digested with restriction enzymes, fractionated on a agarose gel, blotted onto nylon membranes and probed with a ^{32}P-labeled fragment of the human mdmx cDNA.

Results so far do not show any indication for amplification of the mdmx gene in neuroblastoma. To be certain a larger panel of tumors and cell lines has to be analyzed.

Another way to investigate overexpression of the gene is by quantitative RT-PCR analysis of the mRNA expression level in the tumors. The primers mentioned earlier that amplify a specific band of human mRNA could be used for this purpose.

The last method to analyze mdmx expression is at the protein level. Antibodies raised against the protein can be used, if necessary after purification, for in situ histochemistry studies on frozen sections or paraffin-embedded tissues. These types of experiments will give both an answer

as to the level and the localization of the protein in the cell.

Since it is not unlikely that Mdmx (over)expression plays a role in the development in (some types of) cancer, it may be important to be able to block Mdmx function in a cell. If the function is to bind and inactivate p53, this interaction could theoretically be relieved by introducting into the cell an overdose of peptide from the Mdmx domain that is necessary for the association to p53. Through competition the binding will be blocked.

Another possibility is to develop mutants of mdmx that can act dominant-negative on the wild-type protein. Possible target domains for mutations are the putative nucleotide binding site and the putative metal-binding motifs. These mutants can be cloned in viral expression vectors (retrovirus, adenovirus, AAV) for delivery to the target cells. It is still possible that MDMX does not inactivate p53, but actually enhances the functions of p53 or is an intermediate in the p53 functional pathways. If that turns out to be the case, not a mutant but a wild-type mdmx gene will be cloned in the expression vectors mentioned above and be delivered to the target cells.

From the comparison between Mdm2 and Mdmx proteins it can be concluded that some of the amino acids in the p53-binding domain are more important then others. We want to use this knowledge to develop primer sets that might amplify other genes containing a p53-binding domain from the same family.

Experimental

Cloning of the human wild-type p53 sequence into bacterial expression vector

The coding region for the wild-type human p53 protein was amplified by PCR with primers containing a KpnI site at the 5' end and a SacI site at the 3'-end. After amplification and cutting the fragment with the mentioned restriction enzymes the resulting DNA fragment was cloned into a SacI and KpnI-digested modified version of the pET-15b vector obtained from

Novagen. This modified vector contains 5' of the cloning sites a DNA sequence coding for five aminoacids (RRASV) that is recognized as a phosphorylation site for the Heart Muscle Kinase. Downstream from the cloning sites a sequence coding for 6 histidines is present.

As a result a fusion p53 protein can be synthesized in bacteria, containing at its N-terminal end a peptide stretch with a phosphorylation site for the Heart Muscle Kinase (Sigma) and at its C-terminal end a stretch of six histidines that allows purification over a nickel chelate column (Pharmacia). Figure 1 shows the main features of the p53 expression vector.

Production, purification and radioactive labelling of bacterially produced p53 protein

After construction the plasmid was propagated in the bacterial strain HB101, and subsequently transformed into the E. coli B-strain BL21(DE3) or this strain containing pLysE. The induction of protein expression has essentially been performed according to the pET system manual provided by Novagen. In short, a BL21(DE3) colony containing the p53 expression plasmid was grown at 37°C to OD_{600} of 0.6. IPTG was added to a final concentration of 1mM and incubation was continued for another 3 h. After cooling on ice the cells were harvested by centrifugation at 4°C at 5000 x g for 10 min. Cells were grown for additional four hours and pelleted by centrifugation for 10 min at 5000xg. The pellet was rinsed with ice cold PBS and resuspended in IMAC- 5 (20 mM Tris pH 8, 0.5 M NaCl, 5 mM Imidazole, 10 µg/ml PMSF (phenylmethylsulfonyl fluoride). The bacteria were lysed on ice by sonication (3x30 s.) and centrifuged at 20.000xg for 30 min at $4^{O}C$. It turned out that over 90% of the produced p53 protein was retained in the pellet. Bacterial pellet of insoluble proteins including p53 was incubated in IMAC-5 with 6 M urea for 30 minutes on ice. After centrifugation for 30 minutes at 30.000xg the urea was removed from the soluble fraction with the use of a 10DG desalting column (Bio-Rad).

The p53 fusion protein was purified using nickel chelate chromatography according to manufacture instructions (Novagen).

The column was washed with increasing concentrations of imidazole (10,20,40, 100 and 200 nM). Coomassie staining of a SDS-PAGE gel indicated that the protein eluted at 200 nM imidazole was over 70% pure.

For screening an expression library purified protein was radioactively labeled with the HMK(Sigma) and ^{32}p-ATP according to the manufacturers protocol. Unincorporated radioactive nucleotides were removed with the use of a Sephadex G-25 column equilibrated with PBS/1% non-fatty dry milk. The protein could be labeled to a specific activity of > 10^7 cpm per microgram protein.

Screening of expression library with radioactive labeled p53 protein

A cDNA library from a 16-day-old mouse embryo was obtained from Novagen. The cDNAs had been cloned into the lambda EX*lox* vector from Novagen and transformed into the E.coli strain BL21(DE3)pLysE. Approximately 10^6 phages were plated onto 15 150 mm dishes. Dishes were incubated at 37°C for 3-4 hours. When the plaques has become visible, 0.45 µm nitrocellulose filters (Schleicher and Schüll) soaked in 10 mM IPTG were placed over the plaques and proteins were allowed to transfer overnight. Filter hybridization has been performed essentially as described by Ayer et al. (Cell 72, 211-222, 1993). Shortly, the filters were blocked for several hours in HBB/5% NFDM [20 mM HEPES (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 10 mM ß-glycerol phosphate, 1 mM DTT, 0.1% NP-40 and 5% non-fatty dry milk]. Hybridization was performed overnight with at least $2x10^5$ cpm/ml p53 probe in [20 mM HEPES(pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 10 mM ß-glycerol phosphate, 10 mM DTT, 0.1% NP-40 and 10% glycerol] at 4°C. After hybridization the filters were washed once in PBS containing 0.2% Triton X-100 for 10 minutes, and three additional washes were done in PBS, 0.2% Triton X-100, 100 mM KCl, for 15-20 minutes at 4°C.

After washing the filters were allowed to air dry, wrapped in Saran wrap and exposed to X-ray films.

Results: isolation of three cDNAs

The initial screen yielded 10 possible positive clones. After a second and third round screen, only three positive -- lambda clones remained. A purified phage preparation was introduced into the E.coli strain BM25.8 (Novagen). Through the Cre-loxP autosubcloning system, plasmids containing the insert are generated. PCR analysis of the three positive clones with the use of primers from the flanking vector sequences showed inserts of approximately 600, 800 and 1700 bp (named clone 1, 2 and 3, respectively). The first two clones were partially sequenced; the DNA sequence obtained showed no significant homology to any known sequence submitted to the several databases. (Fig 2; partial sequence of two smaller clones, clone 1 and clone 2).

The sequence of the third clone showed clear homology with a cloned human gene called mdm2. This is to date the only human gene whose gene product can bind to and inactivate the tumor suppressor function of p53, probably by inhibiting its capacity to activate the transcription from promoters containing a p53-recognition consensus DNA sequence (see above).

This gene was called the mdmx gene.

The expression of the mdmx gene was investigated by Northern blotting. A polyA+ mRNA blot containing RNA isolated from several mouse tissues was hybridized with a complete mdmx cDNA-fragment isolated. In all tissues mRNAs of approx. 10 kb and 8 kb were observed. In addition, in testis two strong hybridizing bands of ~ 2000 bp and 1700 bp were seen. These bands are also presents in other tissues but much less abundant. It is unclear as yet what the origin of the longer mRNAs is. It is thus not excluded that the genomic gene that gives rise to expression of mdmx codes for several, more or less, related proteins that can or cannot bind to p53.

RNA expression of mdmx could also be found by RT-PCR in several mouse tissues and cell lines. Some of the primers from the mouse sequence did also yield a band of expected length with RNA extracted from human cells/cell lines, indicating already the existence of a human homologue of the mdmx gene. This human PCR fragment was cloned and sequenced. The sequence showed high percentage of identity, but not complete, strongly indicating that part of the human homologue was cloned. In addition, screening of a human cDNA library yielded a cDNA that also has a high percentage identity with the mouse mdmx cDNA. In total approximately 750 bp of the probable human homologue of mdmx has been cloned and sequenced.

To obtain antisera recognizing the Mdmx protein three synthetic peptides were coupled to BSA and injected both in rabbits and in mice. We have now obtained one strongly positive polyclonal rabbit antiserum. Mouse sera will be tested and if positive we will try to generate hybridoma's that produce monoclonal antibodies recognizing mdmx protein.

Expression of mdmx cDNA

The mouse mdmx cDNA was cloned into a CMV-driven eukaryotic expression vector. Transfection of this construct into human of mouse cells yielded the production of an approximately 65 kDa protein as determined by Western blotting on total cell lysates and detection of the protein with a polyclonal rabbit antiserum. (The apparent size of the protein is less than expected based on the homology with mdm2; this runs at approximately 90 kDa; the theoretical molecular weight of both proteins is about 55 kDa. It is not known yet whether the smaller size is an artifact of the expression vector -some mutation?, although we sequenced the expression vector- or that 65 kDa is the real Mr. The fact that the human mdmx protein runs at ~80 kDa on the same type of gel -see below- makes us hesitant about the mouse protein size).

The same expression vector was used to investigate the effect of mdmx expression on transcriptional transactivation by wild-type p53. We found that cotransfection of mdmx could inhibit the transcription activation by wild-type p53, like mdm2. Furthermore, the inhibition by mdmx was mapped to the transcription activation domain of p53 (N-terminal domain). This is the p53 domain to which it was shown that mdm2 binds, suggesting that mdmx also will bind to this part of p53.

Two mouse genomic clones have been isolated from a 129/Ola mouse library. One of these contained the 5' end of the 'cDNA' sequences. To investigate whether upstream sequences contained promoter activity, parts were cloned in front of the luciferase reporter gene. It turned out that 1 kb of upstream sequence is sufficient to obtain full promoter activity. The promoter activity is rather high in this assay, comparable to the RSV Long Terminal Repeat. These results suggest that at least one of the mdmx promoters is located in this region.

Characterization of the same genomic clone indicated a difference in organization compared to mdm2. The mdmx homologues of the sequences that are found in the first three exons of the mdm2 gene. This also means that the p53-responsive element located in the first intron of mdm2 is not

present, at least not in the same position, in the mdmx gene (Fig. 8). The absence of a p53-responsiveness is supported by an experiment showing that mdm2 gene expression is p53-dependently increased after UV-irradiation but that mdmx expression is not increased by the same treatment. This result suggests a different regulation of expression of the mdm2 and mdmx genes. --

Isolation of human MDMX cDNA (hMDMX)

With the use of the mousse cDNA as a probe, a human cDNA-library from T84 colonic epithelial cells was screened to obtain a human cDNA encoding the MDMX protein. Two partial cDNAs were obtained, from which the nucleotide sequence was determined. In Fig. 5 the combined sequence is depicted of the hMDMX cDNA obtained so far. In Fig. 6 a comparison at the protein level between MDMX and hMDMX is shown. In Fig. 7 a comparison between the amino acid sequences of human MDM2 and human MDMX is shown.

The full length coding region of human MDMX was cloned into the modified pET15b vector, described earlier for the production of human p53, and transformed into E. coli BL21. Production of human MDMX protein was induced by the addition of IPTG, cells were harvested and total lysates separated on a polyacrylamide-SDS gel. A protein of approximately 80 kDa was clearly induced upon addition of IPTG. After purification on a nickel-chelate column the protein was injected into rabbits for the production of an antiserum recognizing the human MDMX.

The His-tag in the MDMX-construct in the pET15b expression vector was also replaced by an HA-tag (HA=haemagglutinin). Production of the 'same' 80 kDa protein could now be detected after Western blotting and immunoincubations with the anti-HA antibody.

In addition, the full length coding region for human MDMX was cloned into pcDNA3 vector (Invitrogen). With the use of a coupled in vitro transcription/translation kit (Promega) a ^{35}S-labelled protein was produced of approximately 8- kDa. After incubation with bacterially produced human p53 protein

followed by an anti-p53 immunoprecipitation, this protein was specifically co-precipitated, indicating the binding between human MDMX and human p53. In the same expression vector an HA-tag was added at the carboxy-terminal end of the protein. After transfection into COS-1 cells a protein of approximately 80 kDa could be detected by Western blotting with the use of the commercially available anti HA-tag antibody. --

An internal fragment of the human MDMX cDNA was used as a probe to screen a human P1 genomic library constructed from normal human fibroblast cells (Reference Library - Catabase, Max-Planck-Institut for Molecular Genetics, Berlin-Dahlem, Germany). Finally one P1 clone was purified containing human MDMX sequences. This P1 clone was used for chromosomal localization by FISH. The human MDMX gene was found to localize to human chromosome 1q32. Amplification of this region has been found in some liposarcomas (Forus et al., Genes Chromosomes and Cancer 14, 8-14 (1995); Szymanska et al., Genes Chromosomes and Cancer 15, 89-94 (1996), which might imply a role for MDMX in the genesis of these tumors.